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Combined Effects of *in Utero* and Adolescent Tobacco Smoke Exposure on Lung Function in C57B1/6J Mice

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ABSTRACT

Background: Foetal determinants of airway function, such as *in utero* exposure to maternal cigarette smoke (CS), may create a predisposition to adult airflow obstruction and COPD in adulthood. It has been suggested that active smoking in adolescence and pre-existing airflow obstruction have synergistic deleterious effects.

Objective: We used a mouse model to investigate whether there is a synergistic effect of exposure to CS *in utero* and during adolescence on lung function.

Methods: Female C57Bl/6J mice were exposed to CS or to filtered room air during pregnancy. Exposure to CS began two weeks before mating, and continued until delivery. After birth, the pups were not exposed to CS until day (D)21. Between D21 and D49, corresponding to "adolescence", litters were randomized for an additional 4 weeks of exposure to CS. Lung morphometry, lung mechanics, and the expression of genes involved in senescence were evaluated in different subsets of mice on D21 and D49.

Results: *In utero* exposure to CS induced significant lung function impairment by D21. CS exposure between D21 and D49 induced significant functional impairment only in mice exposed to CS prenatally. On D49, no difference was observed between subgroups in terms of lung p53, p16, p21, and Bax mRNA levels.

Conclusions: Our findings suggest that prenatal and adolescent CS exposure have a synergistic effect on lung function in mice. The combined effect did not appear to be a consequence of early pulmonary senescence.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major cause of death and disability worldwide that is expected to increase in prevalence over the next few years (Lopez et al. 2006). A better understanding of the conditions leading to its occurrence is required to facilitate its prevention. Smoking is the main risk factor for COPD, but only a minority of smokers develops the disease (Chen et al. 2010), and non-smokers account for a substantive part of the burden of COPD (Eisner et al. 2010). Thus, both environmental exposure and individual susceptibility factors are involved in the pathogenesis and progression of COPD.

The quality of lung development is known to play a critical role in determining pulmonary function in adults (Boucherat et al. 2016; Rennard and Drummond 2015). Previous findings suggest that the maximum lung function attained after childhood lung growth is a key determinant of the rate of functional decline leading to a COPD diagnosis (Lange et al. 2015; Rennard and Drummond 2015). Prenatal airway growth may be a key predictor of adult lung function. For example, poor airway function measured shortly after birth in 123 infants predicted airflow obstruction in early adulthood (Stern et al. 2007). A systematic review and meta-analysis by Kotecha et al. (2013) indicated that premature birth, which interrupts physiological prenatal growth, is negatively associated with later pulmonary function in children and adults. Finally, genes involved in early airway morphogenesis have been associated with lung function and COPD (Klar et al. 2011; Van Durme et al. 2010). The impairment of prenatal airway growth may be related to exposure to environmental factors, such as exposure to maternal cigarette smoke (CS) *in utero*. Exposure to maternal CS has been negatively associated with lung function in adolescents and young adults (Hollams et al. 2014) and in early adulthood (Hayatbakhsh et al. 2009). One key question concerns the potential role of pre-existing airway obstruction, secondary to impaired prenatal growth, as a risk factor for an earlier onset or more rapid decline in lung function in individuals beginning to smoke actively in adolescence or early adulthood. Previous studies have suggested that maternal smoking and personal smoking may have a synergistic effect on lung function decline, but were

unable to separate the influences of prenatal and postnatal exposure to parental smoking (Guerra et al. 2013; Upton et al. 2004).

In this study, we used a murine model to investigate whether exposure to cigarette smoke (CS) *in utero* caused an accelerated decline in lung function in mice that were also exposed to CS during adolescence.

Our secondary objective was to determine whether the combined effects of *in utero* exposure and exposure during adolescence to CS led to an acceleration of lung senescence. Several studies have suggested that the ageing process is accelerated in the development of COPD (Ito and Barnes 2009). Pre-bronchodilator FEV1 values were positively associated with telomere length in peripheral leukocytes, a biomarker of cell senescence, in a combined analysis of 14 European cohorts (Albrecht et al. 2014).

METHODS

Animals

Animals

All of the animal experiments were approved by the local institutional animal care and use committee (Agreement n°11/11/15-4), and all animals were treated humanely and with regard for alleviation of suffering. Twelve-week-old female C57Bl/6J mice were obtained from Janvier (France), housed in groups of four and given one week to acclimate to the housing facility. Environmental conditions were a temperature of 21°C, humidity of 55%, lighting of 300 lux (at bench level) and a 12:12 light:dark cycle with lights on at 0800 and off at 2000. Animals were housed in 330x150x130 mm cages and given access to mice maintenance food (ref A03.10, Safe, Augy 89290 France) and water ad libitum. During housing, animals were monitored twice daily for health status. After birth, pups were left in the cage of their mother until day 21. On day 21, litters were separated from their mothers. Males and females were separated and same-sex mice were

randomly housed in groups of eight, depending on the group (Smoke or Air) of their dam.

Animals in the CS exposure group were exposed to filtered mainstream smoke (corresponding to the smoke from 3R4F research cigarettes with filters from the Kentucky Tobacco Research and Development Center, University of Kentucky) 9 cigarettes/hour, 2 hours/day, with a smoking machine (Anitech, Paris, France). Animals were placed in a restraining box and CS was delivered cyclically (1 puff/minute, 15 seconds with the box closed and the remaining 45 seconds with the box open, to mimic typical smoking behaviour as closely as possible). A similar procedure was used for control animals, except that they were exposed to filtered room air rather than CS. Dams were exposed to CS or filtered air for 2 hours/day, 7 days/week, beginning two weeks before mating and continuing through the three weeks of gestation until delivery. For Experiment 2 (see below), a subset of pups were randomized (across litters) to receive CS exposure from D21 through D49, a period corresponding to adolescence [puberty occurs between days 35 and 50 in C57Bl/6J mice (Bates et al. 2003)] (Figure 1). No pups were exposed to CS from birth (D0) to D21, which corresponds to the period of lung growth.

Exposure to CS was monitored through recurrent measurements of blood HbCO levels in dams before mating (pre-conception day 10), during pregnancy (gestational day 5), and in pups two days after the beginning (day 23) and one day before the end (day 48) of the exposure period. Blood (0.2mL) was collected within five minutes following the two-hour CS exposure from an animal randomly selected in each of the five restraining boxes, and blood HbCO levels were assessed on the Radiometer ABL 700 blood gas analyser (Brønshøj, Denmark). The median HbCO levels measured at different time points ranged from 20.9% to 32.5% for dams exposed to CS, and from 24.1% to 31.2% for pups exposed to CS compared with 0.7 to 1.3% and 0.7 to 1.1% for dams and pups exposed to filtered air, respectively (Table S1).

Two experiments were conducted for this study. In experiment 1 (Figure S1) we compared pups with and without *in utero* exposure to CS, including weight and gene expression at D0 (4 litters in each group, 31 and 29 pups, respectively), and weight, gene expression, and lung morphometry at D21 (2 litters and 11 pups in the CS group, 3 litters and 11 pups in the filtered air group). In experiment 2 (Figure S2) we measured weight, lung morphometry, and lung mechanics on D21 in mice with and without *in utero* exposure to CS (14 and 15 pups, respectively), and measured weight, gene expression, lung morphometry, and lung mechanics on D49 in four subgroups of mice:

- Group Air-Air (AA), mice never exposed to CS (n = 12)
- Group Air-Smoke (AS), exposed to CS only between D21 and D49 (n = 19)
- Group Smoke-Air (SA), exposed to CS only during foetal development (n = 11)
- Group Smoke-Smoke (SS), exposed to CS throughout foetal development, and between D21 and D49 (n = 11).

For each experiment and in each group, offspring from at least two different litters were assessed. The experimenters were blinded to the exposure groups while processing data.

Lung mechanics

All functional measures were performed with the FlexiVent (SCIREQ, Montreal, PQ, Canada) - an invasive method that directly measures pulmonary functions via the use of a pre-programmed ventilator and system-specific manoeuvres. This technology measures standard and maximal pressure-volume (PV) curves, resulting in clinically relevant parameters, such as vital capacity, resistance, and compliance of the respiratory system. Data obtained using this method were recently shown to distinguish BALB/c mice models of emphysema and of lung fibrosis from controls (Vanoirbeek et al. 2010). We assessed lung mechanics with the Flexivent system on D21 and D49. Mice were anaesthetised with an intraperitoneal injection of ketamine and xylazine (8.5 mg/kg xylazine and 130 mg/kg ketamine), followed by an additional injection of pentobarbital (40 mg/kg)

to prevent spontaneous breathing. A tracheotomy was performed, and the animals were connected to the FlexiVent system (FlexiVent®, SCIREQ©, Montreal, QC, Canada) and quasi-sinusoidally ventilated with a tidal volume of 10 ml/kg at a frequency of 150 breaths/minute and a positive end-expiratory pressure of 2 cm H_2O. The SCIREQ Flexivent ventilator introduced force oscillation measurements through a graphite piston system that controlled circuit pressure/volume displacements. First, a maximal vital capacity perturbation corresponding to total lung capacity (TLC) was used to obtain maximal inflation of the lungs to a standard pressure of +30 cm H_2O and the lungs were then deflated. A ‘‘snapshot perturbation’’ manoeuvre was then used to measure the resistance (R), compliance (C), and elastance (E) of the whole respiratory system. A forced oscillation perturbation (‘‘Quickprime’’) was used to obtain airway resistance (Rn), tissue damping (resistance) (G), tissue elasticity (H), and tissue hysteresivity (G/H). Finally, maximal PV loops between +30 cm H_2O and -30 cm H_2O were generated to obtain static compliance (Cst) and static elastance (Est). For each parameter, an average of three measurements was calculated and depicted per mouse. A coefficient of determination of 0.95 was the lower limit for accepting a measurement.

Lung morphometry

The left lung was fixed with 4 % paraformaldehyde at a constant hydrostatic pressure of 20 cm H_2O for at least 30 minutes. Lung volumes were estimated by water displacement, as previously described (Scherle 1970). The lungs were dehydrated and embedded in paraffin, and frontal sections were stained with haematoxylin and eosin (H&E). We evaluated a minimum of eight random fields from each left lung of every offspring at x10 magnification by microscopic projection, with the Zen program (Zeiss, Germany). We used the indirect stereological method to quantify mean linear intercept (Lm), lung alveolar surface area, and surface–to-volume ratio, as previously described (Knudsen et al. 2010). The mean linear intercept (L(m)) is commonly used as an index for characterizing the enlargement of airspaces in emphysema (Parameswaran et al. 2006).

In order to study airway remodelling, we used the Sirius Red staining technique (Malkusch et al. 1995) for quantitative morphometric assessment of collagen content. With this technique, collagen fibres are stained bright red and nuclei/cytoplasm are bright yellow. Collagen deposition in isolated bronchi was assessed by colour segmentation (with the "colour deconvolution" plug-in, see Supplemental Material, Image J algorithms) which transformed the RGB (red, green, blue) image into grey scale. Collagen deposition areas that were previously stained red turned black, allowing quantification of the surface area of collagen deposition per unit of the airway basement perimeter.

Gene expression

Previous reports have demonstrated that cigarette smoke induces airway epithelial cellular senescence (Tsuji et al. 2004) and that an accelerated alveolar cell senescence was found in patients with COPD (Tsuji et al. 2006). The expression of genes associated with senescence (p16, p21 and p53) and with apoptosis (p53 and Bax) were studied. p16 and p21 are two senescent-associated cyclin-dependent kinase inhibitors. Their expression is increased in most senescent cells (Krishnamurthy et al. 2004), including alveolar type II and endothelial cells of patients with COPD (Tsuji et al. 2006). p53 is a tumour suppressor which is involved in senescence through the induction of p21, and in apoptosis through the activation of the proapoptotic Bcl-2protein Bax (Chipuk et al. 2004). Mutant mice with activated p53 display an early onset of phenotypes associated with ageing (Tyner et al. 2002).

Levels of p16, p21, p53 and Bax mRNA were analysed at birth, and then on days 21 and 49. Total lung RNA was extracted with the RNeasy Protect Mini kit (Qiagen, France), and reverse transcribed. Quantitative polymerase chain reaction (PCR) was performed in an ABI Prism 7000 Sequence Detection system (Thermo Fisher Scientific, Waltham, MA) with Platinum SYBR Green (Invitrogen, Carlsbad, CA). For each sample, the expression of gene of interest was normalised against the geometric mean value for the housekeeping genes *Sf3a1* and *hprt1*. RNA levels in mouse lung were quantified by a relative quantification method (the ΔΔCT method), as previously

described (Livak and Schmittgen 2001). The primer sequences used are reported in Table S2. One biological sample was used per experimental condition, and each sample was analysed in triplicates.

Finally, lung immunohistochemistry for p16 expression was performed to corroborate the increase in p16 gene expression in smoke-exposed lung detected at day 21 by quantitative-PCR. Sections were prepared and assayed as described in Supplemental Material (Lung immunohistochemistry). Ten digital photomicrographs at × 10 magnification were obtained for a histologic section of each sample. Color segmentation and Image J software with the "colour deconvolution" plug-in (U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016) was used to quantify p16 expression relative to the surface area of the cell nucleus (see Supplemental Material, Image J algorithms)."

Statistical analysis

Data were analysed on an individual dam/pup basis with GraphPad Prism v5.03 (La Jolla, CA). Median values and interquartile ranges are reported. Comparisons were performed with Mann-Whitney tests or Kruskal-Wallis nonparametric analysis of variance tests followed by post-hoc Dunn tests for four groups.

RESULTS

Impact of maternal CS exposure during pregnancy

There was no significant difference in fertility between females exposed or unexposed to CS [13 of 40 (32.5%) and 14 of 40 (35%) found to be pregnant after mating, respectively] (Figures S1 and S2). The median number of offspring per litter also did not differ significantly between CS exposed and air exposed females (9; IQR: 6-10 and 7; IQR: 5-8.5, respectively). In addition, there were no significant differences in maternal body weight at baseline (pre-conception day 14) or on GD0, GD12, or GD18 (Table S3).

Effect of prenatal CS exposure on offspring

At birth, mice that were exposed to CS prenatally (n=31) had significantly lighter body weights than controls (n=29) [median (IQR) of 1.1g (1.1-1.2) and 1.3g (1.3-1.4), respectively ($p<0.001$)] (Table S4). On D21, body weights were not significantly different between pups with (n=47) and without (n=57) prenatal CS exposure [median (IQR) 10.0g (8.6-11.3) and 10.7g (9.4-11.7), respectively]. A subset of twenty-seven mice (17 from the CS group and 10 from the control group) was analysed for lung volume and lung morphometry on D21. Despite a similar median weight in these subsets of mice, lung volume was significantly smaller in CS vs. control mice (Table 1, $p=0.02$).

Alveolarization was evaluated measuring mean linear intercept, alveolar surface area and the surface-to-volume ratio. No significant differences were observed for these parameters between CS and control mice at D21 (Table 1), which suggests that the smaller lung volume at D21 in mice exposed prenatally to CS was not attributable to hypoalveolarization.

Lung function was found to be significantly impaired on D21 in mice exposed prenatally to CS (Figure 2, Table S5 and Table S6). These mice had a significantly lower compliance ($p=0.01$), and a significantly higher elastance ($p<0.01$) of the whole respiratory system than control mice. These results were confirmed by pressure-volume loops, which showed static compliance to be

significantly lower (p<0.01), and static elastance to be significantly higher (p<0.01). Furthermore, the constant-phase model (Quickprime) showed tissue elasticity to be significantly higher (p=0.02) (Table S5). The results obtained were not significantly different between male and female mice exposed to air, or between male and female mice exposed to smoke (Table S7). Resistance was higher in mice with prenatal exposure to CS (median 1.51 cmH2O.s/mL, IQR 1.35-1.65) than in control mice (median 1.31 cmH2O.s/mL, IQR 1.16-1.76) but the difference was not significant (p = 0.47) (Table S5).

Combined effect of prenatal and post-natal CS exposure in mice

On D49, significantly lower body weights were observed in the SS group than in the AA and AS groups (Table S4).

Several lung function values differed significantly between the SS group and the AA and AS groups, whereas there were no significant differences between the AA and AS groups (Figure 2, Table S5 and Table S6). This suggests that effects of CS exposure during adolescence on lung function were limited to mice that also had prenatal CS exposure, consistent with a synergistic effect. Compliance and static compliance values were lowest in the SS group, and elastance and static elastance values were highest in the SS group.

In microscopy, morphometric parameters were not significantly different across the four exposure groups (Table 1). The mean linear intercept was higher and lung alveolar surface area was lower in the SS group than in the other groups, but the difference was not statistically significant (Table 1). Similarly, median collagen deposition was higher in the SS group than the AA group, but the difference was not statistically significant (Table 1 and Figure 3). These findings suggest that associations between CS exposure and reduced lung function were not mediated by emphysema or fibrosis.

Lung expression of senescence genes

Neither prenatal nor postnatal CS exposure was consistently associated with the expression of genes involved in senescence pathways (p16, p21, p53, and Bax). Lung p53 mRNA levels were significantly higher at birth in mice exposed prenatally to CS than in controls (Figure 4A). p53 expression was also higher in SA (n = 11) and SS (N = 11) mice than in AA (n = 7) or AS (n = 16) mice on D49, though the differences were not significant for AA mice (Figure 4C). Lung p16 mRNA levels were low at birth in most mice, similar to the other genes on D49, and high relative to the other genes on D21 (Figure 4 A-C). The median p16 value on D21 was higher in pups exposed to maternal tobacco than in control pups, but the difference was not significant. However, lung p16 protein expression on D21 was significantly higher in mice with prenatal CS exposure than in controls (p=0.02) (Figure S3 C).

Overall, these findings suggest that effects of prenatal and adolescent CS exposure on lung function are not a consequence of early pulmonary senescence.

DISCUSSION

Maternal smoking during pregnancy is known to be associated with poor lung function at birth (Stick et al. 1996), and during adolescence and early adulthood (Hollams et al. 2014; Svanes et al. 2004). Some studies in humans have suggested that parental and personal smoking may have a synergistic effect on the risk of COPD (Guerra et al. 2013; Upton et al. 2004), but a causal link has not been confirmed." To our knowledge, our study is the first to demonstrate evidence of a synergistic effect of prenatal and post-natal CS exposure on lung function in early adulthood in a murine model.

Our mouse model of foetal and adolescent exposure to smoking produced acute HbCO levels in dams and pups that were comparable to those measured after water-pipe smoking in humans (Bentur et al. 2014), and the number of cigarettes pups were exposed to between D21 and D49 was comparable to the consumption of cigarettes by regular young smokers (AIHW 2013; Fuller and

Hawkins 2013). However, our HbCO levels were higher than those observed in regular smokers (Bureau et al. 1982; Pojer et al. 1984; Russell et al. 1976) because in our model, as in most mouse models of tobacco exposure (Fallica et al. 2014; Ng et al. 2006, 2009; Penn et al. 2007; Singh et al. 2013; Wu et al. 2009), mice were exposed to CS continuously for two hours rather than intermittently as in human smokers.

Furthermore, our mice model of foetal exposure to maternal smoking reproduced several consequences of in utero smoke exposure in humans, with lower birthweight and impaired lung function shortly after birth. An analysis of data from almost 17,000 singleton births in England, Scotland, and Wales in 1958 indicated that neonates whose mothers smoked after the fourth month of pregnancy had lower birth weights than other children (Butler et al. 1972), and a study of 663 children reported that lung function parameters measured 2-3 days after birth were lower in children whose mothers smoked during pregnancy, with significant associations in girls (Lødrup Carlsen et al. 1997). Maternal smoking during pregnancy was also associated with lower lung function at age 14 years in a study of 1,127 children (Hollams et al. 2014). Foetal exposure to nicotine has been shown to have many effects on lung structures and lung metabolism (Maritz and Harding 2011). In particular, prenatal tobacco exposure is known to alter airway structure: in a study of 32 infants that died of sudden infant death syndrome (SIDS), the distance between alveolar attachments in the intraparenchymal airways was larger in children whose mothers smoked during pregnancy than in the 8 children whose mothers did not smoke, which suggests that airways may have been narrower in the children exposed to maternal smoking (Elliot et al. 2003) ; in mice, prenatal nicotine exposure has been shown to modify airway geometry in offspring, increasing airway length and decreasing airway diameter, through alpha7 nicotinic acetylcholine receptor (nAChR)–mediated signals (Wongtrakool et al. 2012). We found no significant difference in alveolarisation on day 21 (i.e. at the end of bulk alveolarisation) between mice with and without prenatal exposure to tobacco smoke, but measures of pulmonary function were significantly lower in exposed mice, consistent with impaired airway growth. We observed significantly lower compliance and higher elastance on

D21 in mice with prenatal exposure compared with controls, but there was no significant difference in airway resistance. Tomioka et al. (2002) reported a similar pattern in a mouse model of asthma, in which bronchoconstrictive responses to metacholine and inflammation occurred predominantly at the periphery of the lung, with a significant increase in tissue elastance but not airway resistance.

After a four-week period of CS exposure during a period corresponding to adolescence in mice, lung function parameters were significantly lower in the mice exposed prenatally to CS, but not in mice with adolescent exposure only, when compared with mice that were not exposed during either time period. These findings are consistent with the existence of a synergistic effect between prenatal and post-natal exposure to CS. Because we did not monitor CS exposure chamber conditions such as carbon monoxide or total suspended particulate matter, we could not determine whether there was a threshold effect regarding the amount of exposure needed to induce these alterations in lung function. Synergistic effects of parental and active smoking on lung function impairment have been suggested by human observational studies, but it has not been possible to separate potential effects of prenatal and postnatal environmental factors. Upton et al. (2004) reported that a 10 cigarette/day increase in maternal smoking during pregnancy was associated with significantly lower forced expiratory volume in one second (FEV1)/forced vital capacity (FVC) ratio in current smokers, but not in never smokers or former smokers (Upton et al. 2004). Using the data from the Tucson cohort, Guerra and coworkers found that, at the age of 26, participants with exposure to parental and active smoking had lower FEV1/FVC levels and a steeper decline in FEV1/ FVC between the ages of 11 and 26 than those not exposed to parental or active smoking (Guerra et al. 2013).

The impaired lung function observed in young adult mice was not associated with morphological alterations. In particular, we observed no airspace enlargement compatible with emphysema in the SS group, as attested by mean linear intercept measurements. This may be due to the short duration

of postnatal cigarette smoke exposure, four weeks, whereas at least six months of exposure are usually required to induce emphysema lesions in mice (Rinaldi et al. 2012). In adult mice exposed to CS, lung function impairment also precedes morphological alterations (Rinaldi et al. 2012). In another mouse study, an increase in airway resistance was reported after two months of exposure (Biselli et al. 2011), but we are not aware of any previous studies that measured resistance after only one month of exposure. In the present study we evaluated the combined effects of prenatal CS exposure with adolescent exposure over a 4-week period that was not sufficient to induce lung function impairment in the absence of prenatal exposure. Our findings provide support for the hypothesis that prenatal developmental factors play a role in the early occurrence of COPD in humans. In a clinical trial that enrolled young adult smokers with mild to moderate airflow obstruction, participants with a greater degree of airflow obstruction at baseline had a more rapid rate of lung function decline over time (Drummond et al. 2012). Our findings also suggest that an accelerated decline in lung function may start shortly after the onset of smoking in adolescents at risk (i.e. those with impaired prenatal airway development).

We evaluated various mechanisms that could potentially account for our results. Prenatal and adolescent tobacco exposure did not appear to have a synergistic effect on lung structure, and morphometric measurements did not differ significantly between the subgroups. We evaluated collagen deposition in the airways, as *in utero* exposure to CS has been associated with collagen deposition in the airways of monkey and mice foetuses (Blacquière et al. 2009). No difference was observed between the AA and SS groups. However, it was not possible to study collagen deposition in the small airways. Small airway disease is a key factor leading to airway obstruction in early COPD, and might account for the changes in lung function without morphological changes to the parenchyma observed in the SS group.

We hypothesized that impaired lung function in the SS group might be related to molecular markers of early pulmonary senescence. COPD is often considered to be a disease caused by accelerated lung ageing (Ito and Barnes 2009), and lung ageing is associated with a decrease in small-airway diameter (Niewoehner and Kleinerman 1974) and decreases in FEV1 and FVC (Janssens et al. 1999). As exposure to CS *in utero* causes oxidative stress, a well-known inducer of cell senescence (van Deursen 2014), we hypothesised that lung ageing might begin during prenatal development. However, our results did not support this hypothesis, as p16, p21 and p53 mRNA levels, which were evaluated as molecular markers of senescence in lung tissue, were similar in all groups. Zhou *et al*. (2013) reported that the effect of 6 months of daily cigarette smoke exposure on increased p21 expression and emphysema in female C57Bl6 mice did not differ between mice with exposure beginning at 3 months of age or at 12 months of age, and concluded that the age of the animal had no effect on emphysema development or small-airway remodelling in response to cigarette smoke exposure (Zhou et al. 2013).

Finally, we did not study potential epigenetic changes, which might have accounted for some of our results. *In utero* cigarette smoke exposure has been associated with DNA methylation in humans (reviewed in (Suter et al. 2013)). Cigarette smoking was associated with differences in the methylation patterns of individual genes, including p16, in a study population of non-small cell lung cancer patients and heavy smokers (Destro et al. 2004), and methylation of 7 CpG sites within the p16 gene was significantly correlated in 120 paired maternal-offspring blood samples (Kile et al. 2010). Therefore, lower lung p16 mRNA levels at birth in pups with prenatal cigarette smoke exposure might reflect a transient effect on hypermethylation of the p16 promoter that was not apparent in mice evaluated on D21.

CONCLUSIONS

Our results suggest that prenatal CS exposure and CS exposure during adolescence have a synergistic effect on lung function in early adulthood, consistent with the hypothesis that impaired prenatal pulmonary development is a risk factor for an accelerated decline of lung function leading to COPD in adulthood. In our mouse model, the combined effect did not appear to mediated by an effect of prenatal or adolescent CS exposure on early pulmonary senescence. Our findings support the need for additional research on the molecular mechanisms involved in airway epithelial cell differentiation and alveolar formation at the early stages of life.

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Table 1: Lung volume and lung morphometry of the subsets of mice analysed on these parameters on D21 and D49, with their corresponding weights.

Morphometry	D21			D49				
	In utero exposure to			*In utero* + post-natal exposure to air (A) and/or smoke (S)				
	Air (A)	Smoke (S)	*p*	AA	AS	SA	SS	*p*
	n=10[a]	*n*=17[a]		*n*=10[b]	*n*=17[b]	*n*=8[b]	*n*=8[b]	
Weight (g)	11.0 (9.6-11.8)	10.7 (9.4-12.5)	0.88	18.2 (16.8-21.9)	18.5 (16.7-20.0)	19.0 (16.6-21.3)	16.2 (15.4-17.1)	**0.05**[d]
Lung volume (ml)	0.44 (0.41-0.47)	0.39 (0.34-0.44)	**0.02**	1.8 (1.6-2.2)	1.7 (1.6-1.9)	1.7 (1.6-2.0)	1.7 (1.5-1.8)	0.65[d]
Mean linear intercept (µm)	17.3 (16.6-19.0)	18.6 (16.2-20.0)	0.74	15.4 (15.2-15.7)	16.10 (15.5-16.4)	16.17 (14.7-17.0)	16.40 (15.6-17.7)	0.16[d]
Lung alveolar surface area (cm^2)	84 (71-95)	84 (68-92)	0.94	434 (363-532)	411 (359-443)	422 (352-533)	357 (334-413)	0.20[d]
Surface to-volume ratio (cm^{-1})	285 (263-307)	272 (262-292)	0.41	277 (268-282)	263 (259-283)	271 (248-296)	261 (253-273)	0.57[d]
Collagen deposition[c] (Value/bronchus perimeter (μm^{-1}))	-	-	-	2.83 (1.84-4.12)	-	-	3.5 (2.96-3.66)	0.46

Median values and interquartile range are reported.

a: On day 21, 2 and 4 mice in the in the Control and Smoke group from experiment 1, respectively, and 8 and 13 mice in the Control and Smoke group from experiment 2, respectively, were analysed

for lung volume and morphometry. The results presented in this table on D21 correspond to the pool of these 10 and 17 mice.

b: On D49, all the mice belonged to experiment 2. 10 mice from the group Air-Air (AA), never exposed to CS, 17 mice from the group Air-Smoke (AS), exposed to CS only between D21 and D49, 8 mice from the group Smoke-Air (SA), exposed to CS only during foetal development, and 8 mice from the group Smoke-Smoke (SS), exposed to CS throughout foetal development and between D21 and D49, were analysed for lung volume and lung morphometry. The corresponding weights of these subsets of mice are presented in the Table.

c: Collagen deposition was measured in 8 mice of the SS and 8 mice of the AA groups on D49.

d: Pairwise statistical comparisons were performed and found no statistically significant difference.

Figure 1: Study Plan

The dotted lines indicate periods of exposure to cigarette smoke.

AA group: Air-Air group. AS group: Air-Smoke group. SA group: Smoke-Air group. SS group: Smoke-Smoke group. PC14: pre-conception day 14 (14 days before conception). GD0: gestational day 0 (conception). Pups D0: pups aged 0 day (birth). Pups D21: pups aged 21 days. Pups D49: pups aged 49 days.

At birth, 31 pups were analysed in the Smoke group (weight n=31, gene expression n=24) and 29 in the Air group (weight n=29, gene expression n=21). On day 21, pooling two experiments (Figures S1 and S2), 25 pups were analysed in the Smoke group (weight n=25, lung morphometry n=17, lung mechanics n=14, gene expression n=8) and 26 in the Air group (weight n=26, lung morphometry n=10, lung mechanics n=15, gene expression n=8). On day 49, 11 pups were analysed in the SS group (weight n=11, lung morphometry n=8, lung mechanics n=8, gene expression n=11), 11 in the SA group (weight n=11, lung morphometry n=8, lung mechanics n=8, gene expression n=11), 19 in the AS group (weight n=19, lung morphometry n=17, lung mechanics n=8, gene expression n=16), 12 in the AA group (weight n=12, lung morphometry n=10, lung mechanics n=8, gene expression n=7).

Figure 2: Lung mechanics on days 21 and 49.

Results are presented as the median and IQR. *: $p < 0.05$; **: $p < 0.01$

D21: day 21; A: air group (n=15); S: smoke group (n=14).

D49: day 49; AA: air-air group; AS: air-smoke group; SA: smoke-air group; SS: smoke-smoke group. (n=8 per group).

Numeric data for this figure are available in Supplemental Material, Tables S5 and S6.

On day 21, medians were compared using a Mann-Whitney test. Difference were statistically significant between Smoke and Air groups regarding compliance (p=0.01), elastance (p<0.01), static compliance (p<0.01) and static elastance (p<0.01).

On day 49, medians were compared using a Kruskal-Wallis test followed by post-hoc Dunn tests for four groups. Overall p-values (not shown on the figure) were < 0.01 for compliance and elastance, and < 0.05 for static compliance and static elastance. Post-hoc Dunn tests revealed differences statistically significant between the SS group and the AA and AS groups for compliance and elastance ($p<0.05$), and between SS and AS groups for static compliance and static elastance ($p<0.05$).

Figure 3: Collagen deposition in the main bronchi

Histologic analysis of lung parenchyma of 49-day-old mice A. from the group Air-Air. B. from the group Smoke-Smoke. Collagen deposition in the airway wall was visible on picro-sirius red staining (arrow). a: alveolus; A: artery; B: bronchus: V: vein. C. Collagen deposition in the main bronchi did not differ significantly between the AA and SS groups (n=8 per group).

Figure 4: Expression of the p16, p21, p53 and Bax genes at birth, and on D21 and D49. Results are presented as the median and IQR.

A. Gene expression at birth after prenatal exposure to air (Group A) or CS (Group S). Group A, n=21; Group S, n=24; *$p<0.05$

B. Gene expression on day 21(D21) after prenatal exposure to air (Group A) or CS (Group S); n=8 per group.

C. Gene expression on day 49 (D49) in mice never exposed (AA, n=7), exposed to CS during postnatal life only (AS, n=16), exposed during prenatal development only (SA, n=11), and exposed during prenatal development and postnatal life (SS, n=11), *$p<0.05$

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Figure 1.

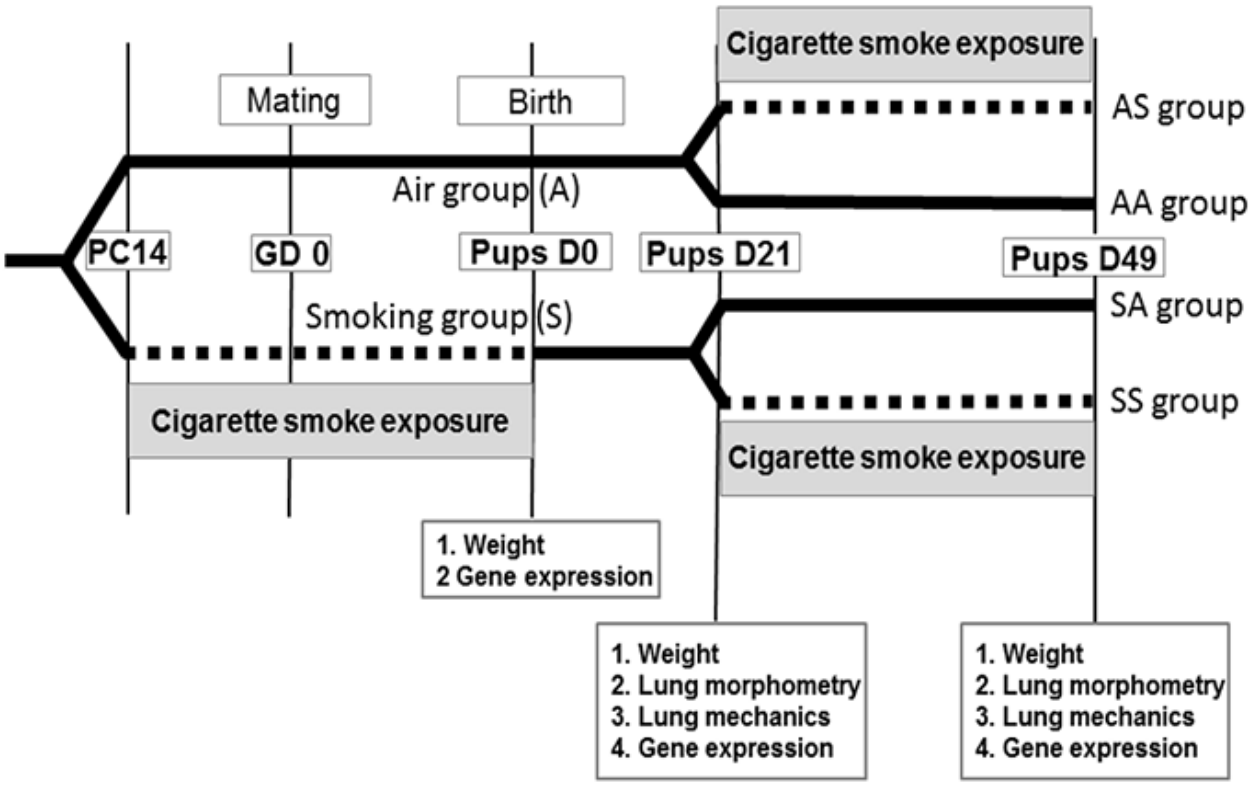

Figure 2.

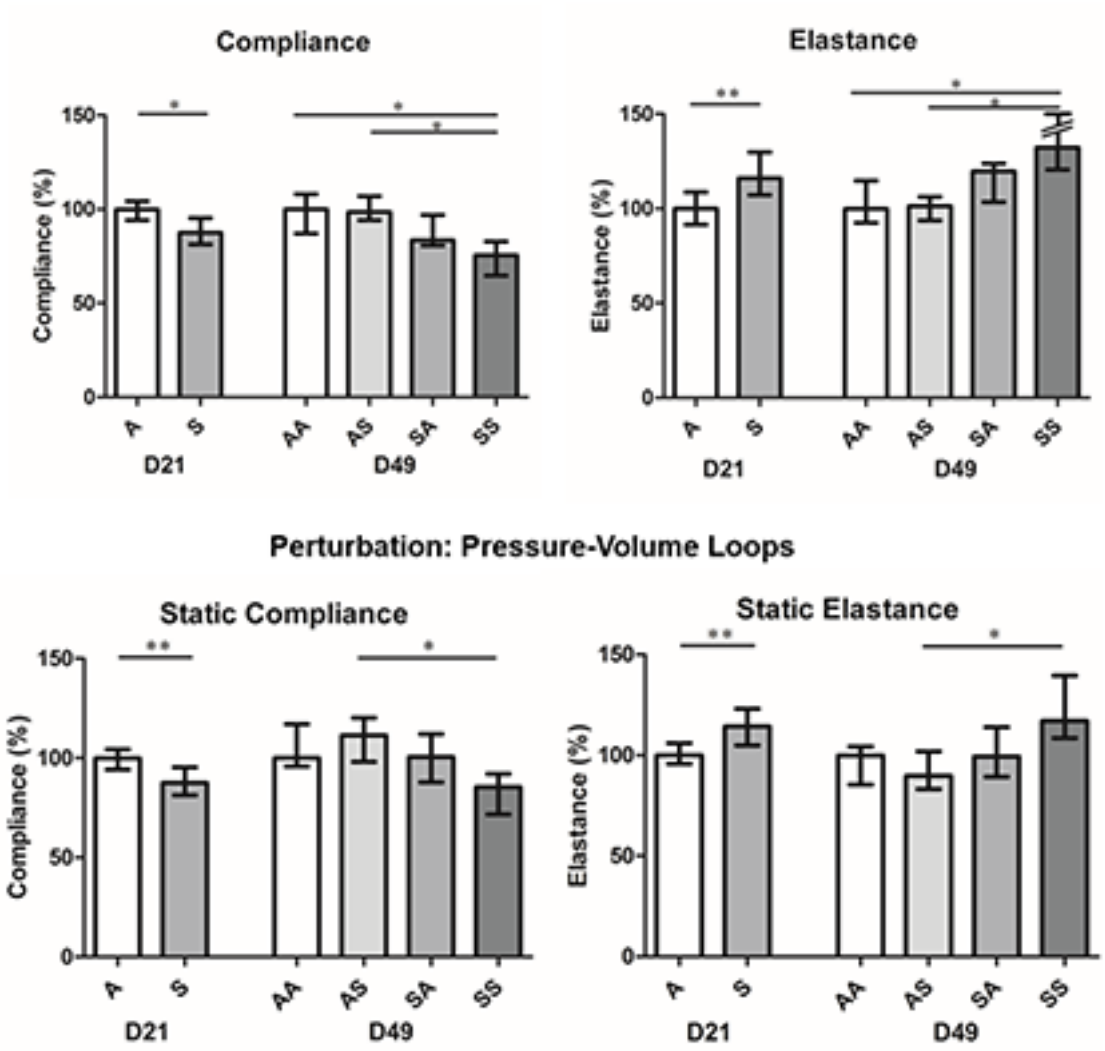
Compliance
Compliance (%)
150
100
50
0
A
S
AA
AS
SA
SS
D21
D49
Elastance
Elastance (%)
Perturbation: Pressure-Volume Loops
Static Compliance
Static Elastance

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Figure 3.

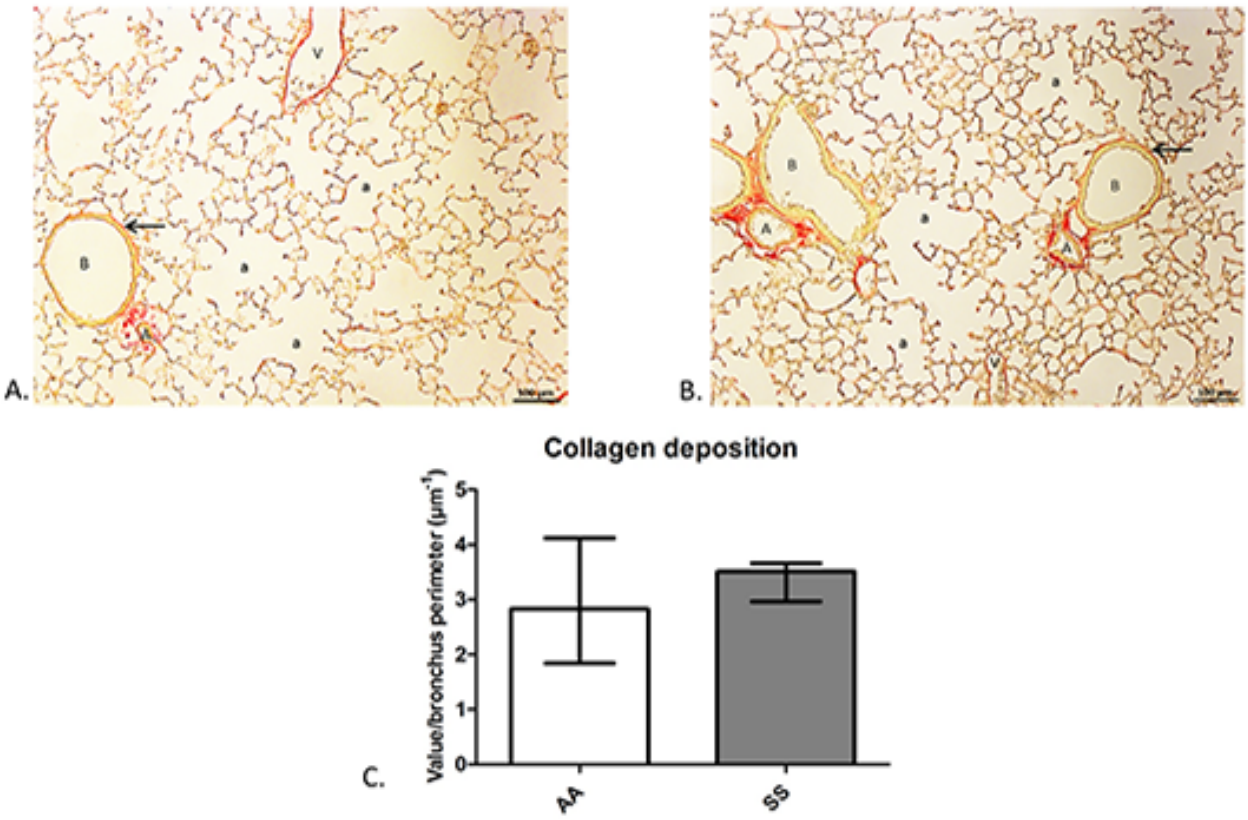
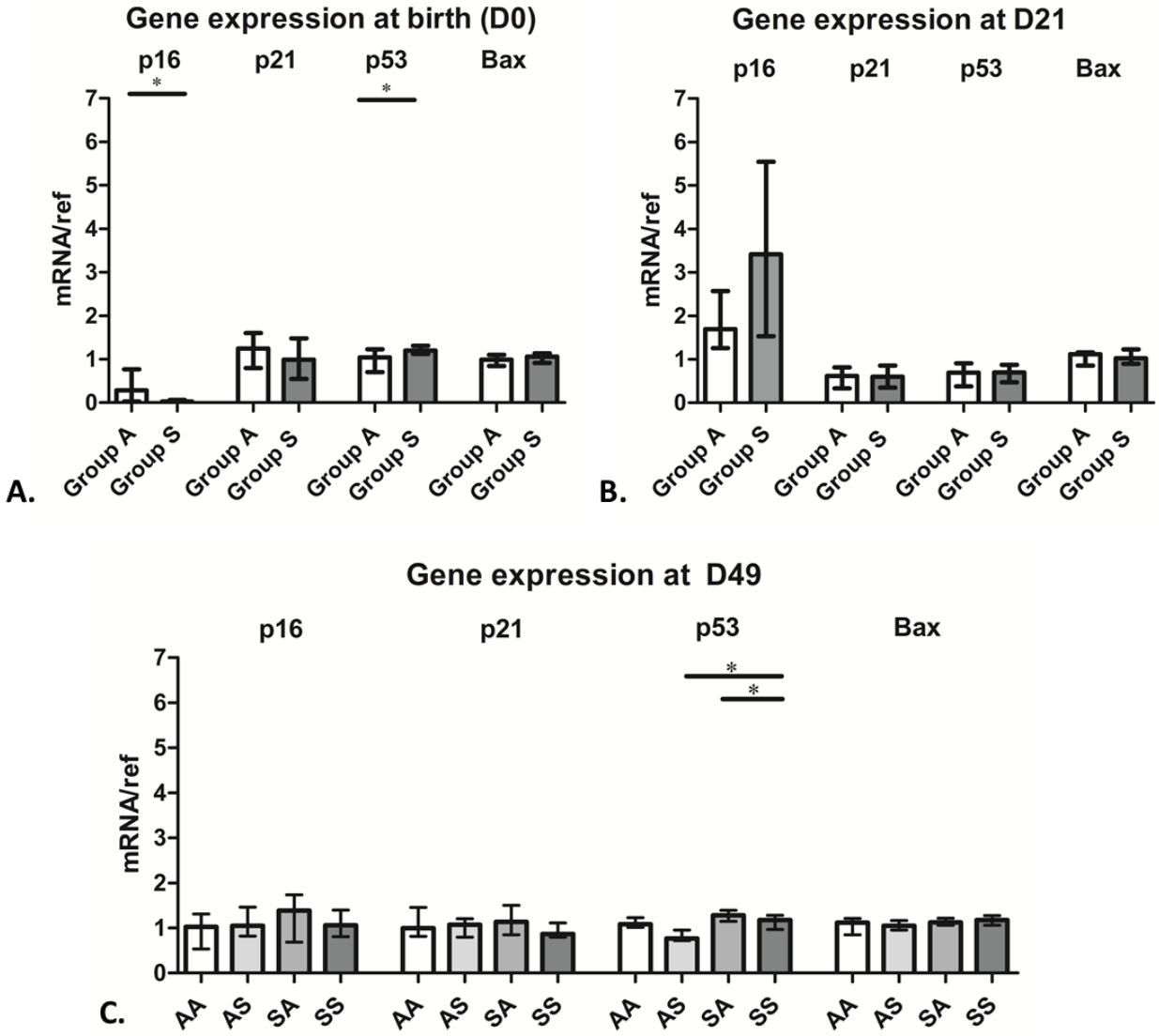


Figure 4.


Gene expression at birth (D0)
p16
p21
p53
Bax
mRNA/ref
Group A
Group S
A.
Gene expression at D21
B.
Gene expression at D49
AA
AS
SA
SS
C.